Employing this approach, simultaneous testing of biological materials for the presence of virulence genes of Lcoh strains and other enterobacteria, harboring virulence genes can be semiautomated and performed within 18 h. --

Please delete the paragraph on page 5, lines 15-19, and substitute the following paragraph:

-- According to the present invention Real Time PCR (e.g., TAQMAN™ PCR) for the detection of pathogenic *E.coli* is provided, enabling for the first time the specific, rapid and high throughput routine detection of EHEC, ETEC, EPEC, EIEC, and EaggEC and related enterobacteria that harbor these virulence genes in routine bacteriological laboratories. --

Please delete the paragraph on page 16, lines3-12, and substitute the following paragraph:

-- various post-PCR steps in order to verify whether specific amplification of a target gene has occured (68J2). The TAQMAN™-PCR detection system (74,75,90) enables the rapid, specific, sensitive, and high-throughput diagnosis for differentiation of pathogenic Lcoli strains from other strains of Ecoli. The assay has the ability to quantify the intial target sequence. Since PCR-reaction tubes have not to be opened after PCR cycling, the potential danger of cross-PCR contamination is almost neglegible. The scanning time of 96 samples is approximately 8 min, and calculation of test results can be automated with a commercially available spred sheet program. Thus, overall post-PCR processing time is cut to a minimum.--

Please delete the paragraph on page 16, lines14-30, and substitute the following paragraph:

-- The TAQMAN™ -System relies on standard PCR technique with the addition of a specific internal fluorogenic oligonucleotide probe. The combination of conventional PCR with the Taq polymerase-dependent degradation of an internally hybridized ohgonucleotide probe confers also specificity to this detection method, since it is highly unlikely that unspecific PCR amplification will yield positive fluorescence signals. Some rules for chosing the fluorigenic probes have to be obeyed (74,75). Criticial are the lenght of the probe, the location of reporter and quencher dyes and the absence of a guanosine at the 5'-end (74). Also, the distance of the

probe from one of the specific PCR primers is important. This is due to the fact that the probe has to stay annealed to the template strand in order to be cleaved by Taq polymerase. Since annealing depends, at least partially, on the Tm of the probe, probes should be designed to have a higher Tm as the primers. According to the present invention this was solved (except for sltll) by designing probes that were 3 to 6 bp longer than the specific primers. PCR amplification includes extension of the target sequence after annealing of the primers and the Tm of the extended primers increases. For ...--

Please delete the paragraph on page 17, lines 2-15, and substitute the following paragraph:

-- the fluorogenic oligonucleotide probe, where the 3'- end is capped in order to avoid elongation, the Tm remains constant, making it more likely that the probe dissociates before degradation by Taq polymerase. Oligonucleotide probe degradation can be optimized by spatial proximity of the fluorogenic probe and the primer. By moving the probe for sltI from 121 bp to 9 bp close to the primer, a significant improvement in ARQ values could be obtained. A second strategy of optimization of TAQMAN™-PCR is to perform PCR elongation at 65^0C, where it is also less likely that the probe dissociates from the template strand before Taq polymerase reaches and hydrolizes it. Values for ΔRQ can thus again be increased about 1.2 to 1.5 fold. The increase of ΔRQ values might be due to the ratio of annealed oligonucleotide probe reached by Taq polymerase or to an increased processivity of Taq polymerase.--

Please delete the paragraph on page 17, lines 17-30, and substitute the following paragraph:

-- The concentration of fluorogenic probes influences the accuracy of TAQMAN™ - results. When the probe concentrations were > 50 pmol / PCR reaction only a relatively small fraction was hydrolysed by Taq polymerase. The ratio of undegraded probe to degraded probe remains high and the fluorescence emmission of the unquenched reporter dye does not significantly increase in relation to the fluorescence intensity of the reporter dye sill close to the quencher. Thus, at high probe concentrations, ΔRQ values are lower than with intermediate probe concentrations (10 20 pmol). When the probe concentration is too low, ΔRQ values are increased, however, variability of PCR results is increased, since probably small errors in

pipeting or minimal differences between PCR reactions become critical. Optimal probe concentration that yielded smallest variabilties and highest RQ values were found at a probe concentration of 20 pmol.--

Please delete the paragraph on page 18, lines 3-15, and substitute the following paragraph:

-- Since TAQMAN™-PCR uses an internal oligonucleotide probe for detection of template amplification, specific primers and probes can be amply designed. The design of primer and probe sequences is especially important, when nucleotide sequence variants of a given gene exist. This is the case for sltI and sltII. For sltI, all published sequences were aligned and primers and probes were designed to bind to conserved regions of all three variants. For sltII, only one region of the published genes was conserved, thus this region was chosen for the fluorogenic oligonucleotide probe. The primers for amplification of sItll were designed to contain all possible nucleotide sequences at the ambiguous positions of the published sltll variants (degenerate primer approach) (79-83). By employing degenerate primers, it is possible to detect all published variants in one single PCR reaction.--

Please delete the paragraph on page 18, lines 17-26, and substitute the following paragraph:

-- The isolation method for template DNA affects the performance of the PCR. Two methods, that are suited as rapid purification steps for routine applications, namely boiling prep or spin prep were compared. Boiling preps may still contain some bacterial components that can affect PCR reactions, however, it is extremely fast. The spin prep method involves isolation steps that serve to purify DNA from potentially negatively influencing materials. ΔRQ values and sensitivity of TAQMAN™-PCR for virulence genes from enterobacteria was not found significantly increased as compared to boiling preps when template DNA was prepared by spin prep method.--

Please delete the paragraph on page 17, lines 28-30, and substitute the following paragraph:

C9

-- The overall sensitivity of TAQMAN™-PCR for all primer/probe combinations was comparable to visual scoring of PCR products by detection with ethidium bromide stained agarose gel electrophoresis. Under optimized...--

Please delete the paragraph on page 20, lines 3-21, and substitute the following paragraph:

-- flora". In order to address this problem a set of specific primers and fluorogenic probes were developed and optimized for TAQMAN™ -based detection of virulence factors harbored by these bacteria (Tables 2 and 3). Arranging patient samples, positive and no-template controls of all 8 tested virulence genes in a standard 96 well microtiter format, a turnaround time from preparation of sample DNA to fluorescence measurement of under 5 hours can be achieved. Thus, the TAQMAN™ -based assay for pathogenic *E.coli* provides an ultrarapid means of diagnosis of these bacteria. While being accurate, sensitive and specific, this assay requires minimal post PCR processing time compared to conventional methods. When TAQMAN™-PCR is performed in optical tubes also the danger of cross-contamination of PCR reactions with amplified products is reduced to a minimum. Detection of virulence plasmids harbored by pathogenic enterobacteria might prove the potential of these bacteria to cause disease in the host. It is not clear whether enterobacteria that contain toxin genes or attachment factors do also always express them outside the host. This might be an explanation why ELISA tests for shiga like toxins might be negative in a number of HUS cases where sltI and/or sltII containing EHECs can be detected by nucleic acid based methods.--

C10

Please delete the paragraph on page 20, lines 3-30, and substitute the following paragraph:

-- The TAQMAN™ -assay according to the invention for detection of pathogenic Ecoli was then tested in a routine diagnostic setting for the examination of stool samples obtained from children with diarrhea within a defined geographic area (Southern Bavaria) during a 7 month period. Results obtained by TAQMAN™-PCR were compared to the standard detection method for PCR products (electrophoresis of ethidium stained agarose gels). 100 stool samples were analysed (Table 4). 22% of samples were found to test positive for one or more virulence factors. There were 2 cases....--

C11

Please delete the paragraph on page 21, lines14-24, and substitute the following paragraph:

C12

--Collectively, these investigations show that a large proportion of diarrheal diseases in children and also in adults are associated with pathogenic *E. coli* that are falsely diagnosed as commensal flora in standard microbiological procedures. The TAQMAN™ methodology according to the invention for the first time enables the direct, fast, specific, and sensitive detection of these important pathogens. Moreover, virulence genes detected with this approach are not confined to *E.coli*, they also can be freely transmitted to other enterobacteria. Detection of the virulence genes within these bacteria would also be covered by the herein described TAQMAN™-PCR The assay requires only minimal post-PCR detection time, can thus be performed under 18 hours, and abolishes PCR-cross contamination problems. --

Please delete the paragraph on page 22, lines 27-30, and substitute the following paragraph:

C13

-- TAQMAN™-PCR was optimized by isolation of DNA from *E.coli* control strains harboring genes for LT, ST, *inv*-plasmid, pCVD342, EAF, *eae*, sltI and sltII (see Table 1). $MgCl_2$ concentrations were adjusted for maximum PCR..--

Please delete the paragraph on page 23, lines 24-30, and page 24, lines 3-6 and substitute the following paragraph:

C14

--The influence of DNA preparation on the performance of TAQMAN™-PCR was tested, since it has been reported that crude bacterial lysates can contain inhibiting factors that might interfere with PCR performance. Therefore, bacteria were collected after overnight growth on McConkey plates. DNA was prepared by boiling of bacteria inoculated in 0.9% NaCl solution or by isolation of genomic DNA with a commercial spin prep procedure (see the example, material and methods). The RQ values and sensititvity of TAQMAN™-PCR did not differ when the two preparation methods were compared. The RQ values obtained for PCR amplifications from DNA derived from 10^5 Sltl or sltII containing EHEC prepared by boiling or by spin prep comparable. --

Please delete the paragraph on page 24, lines 8-15, and substitute the following paragraph:

--The TAQMAN™-PCR method relies on the detection of free reporter dye (FAM) that is released from the probe after hydrolysis. Thus, probe concentration should also have an effect on the assay performance by affecting the fraction of the probe that is degraded during PCR cycling. Probe concentrations were titrated in the range of 100 pmol to 0.1 pmol and ΔRQ values were determined. Optimal probe concentrations varied in between 10 pmol and 20 pmol depending on the target gene that was amplified. --

Please delete the paragraph on page 24, lines 17-30, and page 25, lines 3-4 substitute the following paragraph:

-- For testing sensitivity of TAQMAN™-PCR , EHEC containing either sltI or sltII were diluted in a suspension containing E.coli strain ATCC11775 at 10^7 CfU at log step dilutions. PCR was performed under optimized conditions and results from ethidium-bromide stained agarose gels were compared to TaqManTm results. Minimum detection limits of a sltI containing EHEC strain was 10^3 cfu within 10^7. For sltII the detection limit was found at $10^{3.5}$ cfu in 10^7 enterobacteria. Both methods, detection of PCR products by agarose gel electrophoresis and measurement of fluorescence signals by the TaqMan method yielded comparable results, i.e. that at ΔRQ values above $\Delta RQ_{threshold}$ PCR product bands were visible in agarose gelb whereas at ΔRQ values around $\Delta RQ_{threshold}$ also in agarose gels PCR products were below the detection limit. After optimizing detection tests for all virulence factors/ toxins, TAQMAN™-PCR was set up for routine testing of biological specimen for the presence of pathogenic E.coli bacteria. Results of TAQMAN™-PCR were compared to agarose gel electrophoresis. . –

Please delete the paragraph on page 25, lines 15-19, and substitute the following paragraph:

-- In order to verify TAQMAN™-PCR performance and to test for the occurence of pathogenic E.coli screening of 100 stool specimens from children of age 0 to 10 years with the clinical symptoms of diarrhea was undertaken. The materials and methods used in the test are described in more detail below under item 2.--

Please delete the paragraph on page 26, lines 17-23 and substitute the following paragraph:

-- Enterobacteria from the two patients with EHEC were hybridized with sltl and sltII gene probes for testing accuracy and specificity of TAQMAN™-PCR. In the case of patient one, where TAQMAN™-PCR was positive for sltl, only colonies hybridizing with sltI could be found. Colonies of patient two, where TAQMAN™-PCR was positive for sItI and sltII, hybridized with probes for sltI and sltII. Positive colonies were picked and biochemically typed as *E. coli*--.

C18

Please delete the paragraph on page 29, lines 3-11, and substitute the following paragraph:

-- e) Sensitivity of TAQMAN™ technique: For determination of the sensitivity of the TAQMAN™ method, serial log-step dilutions of positive control strains were performed in a solution containing 10^7 cfu of Ecoli reference strain ATCC 11775 DNA was either prepared by the boiling method (see above) or purified using spin prep columns designed for isolation of genomic bacterial DNA (Qiagen, Germany). Purification was according to the protocol of the manufacturer. The detection limit for sItI containing strains was determined with 10^3 cfu among 10^7 E.coli and for sItII containing strains as $10^{3.5}$ among 10^7.

C19

Please delete the paragraph on page 29, lines 13-31 and substitute the following paragraph:

-- f) Colony hybridisation and isolation of EHEC bacteria: EHEC bacterial strains and stool samples from patients testing positive in sltI or sItII TAQMAN™-PCR were subjected to colony hybridisation. Briefly, bacteria were plated on McConkey agar plates such that single colonies could be seen. Bacteria were blotted on nylon membranes (Genescreen Plus, NEN, Germany), cracked (1% SDS), denatured (0.5M NaOH, 1.5M NaCI), neutralized (1M TRIS, 1.5M NaCI), and washed (20xSSC). Membranes were baked at 80^0C for 2 hours. DNA probes specific for sal or sltII were labelled with fluorescein (Gene-Images random prime labelling module, Amersham, Germany). Afterwards, filters were hybridized with labelled probes. Hybridization was verified by non-radioactive detection system employing anti-FITC peroxidase mAb and ECL detection module (Gene Images CDP-Star detection module, Amersham, Germany). Bacterial colonies hybridizing with the probe and non-hybridizing colonies were picked, verified by TAQMAN™-PCR and tested for antibiotic susceptibility. Antibiotic

C20